

*Contributions to a better understanding of
morphogenesis in Picea abies (L.) Karst.
tissues cultured in vitro*

Eva Jansson



Lund 1983

COVER: Embryonic shoot of *Picea abies* (Norway spruce) with adventitious buds induced in vitro in needle primordia.

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Contributions to a better understanding of
morphogenesis in *Poa annua* (L.) Karst.
tissues cultured in vitro

Eric Jorgensen



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June 1983

CONTRIBUTIONS TO A BETTER UNDERSTANDING OF MORPHOGENESIS
IN PICEA ABIES (L.) KARST. TISSUES IN VITRO

by

Fil.kand. Eva Jansson (Ög)

Academic dissertation

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Abstract In vitro propagation of Picea abies (L.) Karst. from needles of 20-22-week old plants or embryonic shoots plus or minus crown from 10-year-old trees was investigated. Shoot buds and shoots could be induced when explants were treated with cytokinins. A higher frequency of meristemoids were initiated, and their subsequent differentiation into shoots was stimulated on needle explants with the addition of nanomolar levels of auxin in combination with micromolar concentrations of cytokinins. The formation of adventitious structures generally decreased with increasing needle age and distance from the needle base. Cell divisions were initiated in both epi- and hypodermal layers, whereas more distally, they were often restricted to the epidermis and subsidiary cells of the stomatal complexes. Tissue proximal to the abscission zone retained its organogenic property longer than the rest of the needle, illustrating the importance of the manner in which needles are excised. Bud-induced embryonic shoots plus crown responded better than those lacking it, both with respect to the number of explants producing adventitious shoots and the numbers of adventitious shoots induced per explant. Anatomical events were studied during the course of the experiments. By performing transport studies using radiolabelled isotopes, the crown was found to prevent both acropetal and basipetal transport of substances such as [^{32}P] and [^{14}C]-IAA.			
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INTRODUCTION

The fact that *Picea abies* (Norway spruce) attains sexual maturity at an age of 30 to 60 years (Schmidt-Vogt 1978) and that flowering is highly irregular, means that the time involved in producing successive generations of improved seed is considerable. Most forest trees are difficult to propagate vegetatively by rooted cuttings, therefore plants needed in reforestation programmes are more easily produced from seed provided by clonal seed orchards established by grafting (Sommer and Brown 1979).

The predicted rising demand for forest products in the world has led to the development of intensified tree improvement programmes demanding new, effective and rapid methods of propagation. Since at least part of the genetic variation due to gene interactions not normally transmitted intact through sexual reproduction can be secured by either, long-term interbreeding of homozygous trees or relatively short-term clonal vegetative propagation, much attention is now being paid to the so-called clonal option (Libby 1979). Multiplication of *Picea abies* via rooted cuttings (Kleinschmidt 1977) is being undertaken on an ever-increasing scale in Sweden (3 million estimated for 1983).

With regard to vegetative propagation, there is a serious problem that remains to be solved before mature trees old enough to have demonstrated their superior characteristics will be able to be cloned. This is the

problem of maturation in which is manifested for example, rapid loss in rooting capacity, plagiotrophic growth habit, and decline in growth and survival. As realized by all propagators of the conifer, whether using in vitro or in vivo techniques and as pointed out by Romberger (1976) and Biondi and Thorpe (1981), maturation is the key obstacle to successful vegetative propagation of the superior tree.

Conifer tissues were among the first to be cultured in vitro (Gautheret 1934). Ball (1950) first reported the differentiation of adventitious buds in somatic callus tissues of *Sequoia sempervirens*.

The discovery of the cytokinins (Miller et al. 1955) and their influence on organogenesis in somatic tissues greatly accelerated the development of tissue culture techniques for vegetative micropropagation. The concept of Skoog and Miller (1957) of the cytokinin/auxin balance determining the direction of morphogenesis has withstood the test of time fairly well and seems to be valid also for a large number of forest tree species (Zaerr and Mapes 1982).

The production of conifer plantlings using in vitro techniques was reported by Cheng (1975) and also by Sommer et al. (1975), both working with embryonic tissues of *Pseudotsuga menziesii* and the latter also with those of *Pinus palustris*. The tissues were subjected to media containing high concentrations of cyto-

kinins, and responded by forming adventitious buds which after developing into shoots, could be rooted.

Since 1975 interest in the culture of conifers in vitro has expanded very rapidly with varying degrees of success in plant regeneration. To list some examples: Campbell and Durzan (1976), *Picea glauca*; Reilly and Washer (1977), *Pinus radiata*; David and David (1977), *Pinus pinaster*; Chalupa (1977), Von Arnold and Eriksson (1978, 1979a,b) and Bornman (1983), *Picea abies*; for an extensive review see David (1982).

Most of this regeneration was performed with juvenile material and only in a few cases were tissues from mature coniferous trees successfully cultured. As far as the Pinaceae is concerned the first such record was that of 11-year-old *Pinus pinaster* (David et al. 1979).

With the problems experienced in the recalcitrant nature of gymnospermous tissues coupled with the application of techniques essentially developed for angiospermous callus, it is obvious that much fundamental work is still required to help in elucidating the many-faceted problems.

The aim of this investigation at the outset was to explore whether it were possible to propagate *Picea abies* from mature tissues using in vitro culture techniques. However, it was soon realized that several problems associated with the micropropagation of this conifer and presumably also others required more funda-

mental research. A closer consideration of the anatomical constraints associated with inductive events in vitro was therefore necessary in view of the fact that the organogenic response varies with needle age, the manner in which tissues are explanted, the position on the needle's axis where adventitious structures differentiated (II), etc.

It was reasoned that by studying structural or anatomical changes, the observed phenomena could be more meaningfully interpreted functionally or physiologically. A case in point is the crown of the embryonic shoot, the retention of which in the explant was reported to stimulate shoot growth in vitro (Chalupa and Durzan 1973). The role of the presence or absence of this morphological structure on the de novo induction of adventitious shoots (III) was therefore investigated, as was also its possible role as a translocation barrier (IV).

Abbreviations - ABA, abscisic acid; AZ, abscission zone; BA, N⁶-benzyladenine; FW, fresh weight; IAA, indoleacetic acid; NAA, 1-naphthaleneacetic acid.

This thesis is based on the following publications:

- I Jansson, E. & Bornman, C.H. 1980. In vitro phyllo-morphic regeneration of shoot buds and shoots in *Picea abies*. - *Physiol. Plant.* 49: 105-111.

- II Jansson, E. & Bornman, C.H. 1981. In vitro initiation of adventitious structures in relation to the abscission zone in needle explants of *Picea abies*: anatomical considerations. - *Physiol. Plant.* 53: 191-197.
- III Jansson, E. & Bornman, C.H. 1983. Morphogenesis in dormant embryonic shoots of *Picea abies*: Influence of the crown and cold treatment. - *Physiol. Plant.* 59: 1-8.
- IV Jansson, E., Jensen, P. & Bornman, C.H. 1983. Effect of the crown on uptake and transport in embryonic shoots of *Picea abies*. - *Physiol. Plant.* 59: 521-527.

EXPERIMENTAL PROCEDURE

Needles from newly-flushed, frost-hardened 20-22-week-old plants (I,II) or dormant embryonic shoots plus or minus crown from 10-year-old trees (III,IV) of *Picea abies* (L.) Karst. comprised the plant material. Needles and embryonic shoots were cultured on an agarified culture medium in petri dishes or erlenmeyer flasks, respectively.

The bud-induction treatment in the form of BA and NAA or BA alone was given through the medium (I,II) for 6 weeks or as a 3-h-pulse (III) after the plant material had been disinfested. After the bud-induction period the

explants were transferred to fresh media every third or fourth week and screened weekly. The explants were cultured in a climate chamber at $20 \pm 1^\circ\text{C}$ under a 16-h photoperiod (irradiance ca 17.5 W/m^2).

Material for anatomical studies (II,III) was prepared histologically using either paraffin-wax or plastic embedding techniques.

Transport experiments (IV), using radiolabelled substances ($[^{14}\text{C}]$ - IAA, $[^{32}\text{P}]$ - phosphate, $[^{35}\text{S}]$ - sulphate and ^{86}Rb as a tracer for K^+) supplied in an agarified medium modified from that of Schenk and Hildebrandt (1972), were performed as shown in Fig. 1. Radioactivity was determined by liquid scintillation spectrometry.

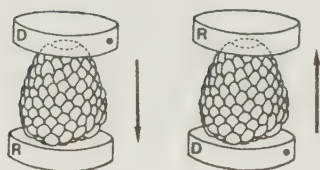


Fig. 1. Line drawings illustrating the positioning of agar blocks. R, receiver and D, donor in relation to the embryonic shoot used in transport experiments. Arrows indicate principal direction of transport.

RESULTS

I *In vitro* phyllomorphic regeneration of shoot buds shoots in *Picea abies*.

Shoot buds and shoots along with other morphological structures such as phylloids or leaflike projections, nodules and also calli were initiated on needle explants cultured on media supplemented with BA and NAA (Fig. 2).

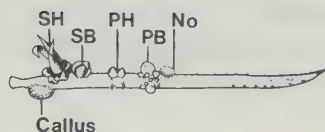


Fig. 2. An illustration of the different types of morphological structures that differentiated in response to various treatments on the needle explant. SH, shoot; SB, shoot bud; PH, phylloid; PB, pseudobulbil; No, nodule.

The cytokinin to auxin concentration in the induction medium was important in determining the organogenic response. The best response regarding adventitious shoot formation was obtained on a medium containing $5\mu\text{M}$ BA plus 50 nM NAA, where up to 22% of the needle explants responded. With increasing NAA concentrations, 0, 5 and 50 nM in combination with 5 or $10\mu\text{M}$ BA, the induction of adventitious structures commenced in a more distal direction (Fig. 3), and the increasing NAA concent-

rations also had stimulatory effects on shoot development.

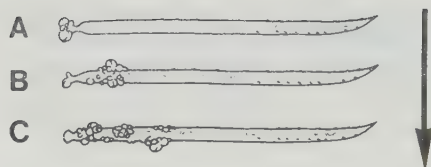


Fig. 3. Using either 10 or 5 μ M BA in combination with increasing concentrations of NAA, pseudobulbils tended to form in a progressively more distal position on the needle explant. Arrow indicates increasing concentration of NAA. A, 0; B, 5; and C, 50 nM NAA.

When the auxin to cytokinin ratio was 1:1, there was only callus formation. Phylloids, shoot buds or shoots were observed when the level of exogenously applied auxin was kept at the nanomolar level.

The initial development was usually very slow, and not until after 16 weeks in culture could the first well-developed shoots be observed, frequently two to three per explant. Occasionally adventitious buds formed after only 6 weeks, but they were always restricted to the base of the needle proximal to the abscission zone.

The degree of differentiation on the phyllomorph explant usually decreased acropetally; that is, near the base shoot buds and shoots were produced, while distal to

these, phylloids, undeveloped pseudobulbils and nodules developed.

In conclusion, one can assume that hormone gradients exist within a single young needle in its active state of growth and development. Therefore the level of endogenously applied auxin and cytokinin might be a factor influencing both the induction process of adventitious structures and their position on the needle axis.

II In vitro initiation of adventitious structures in relation to the abscission zone in needle explants of *Picea abies*: anatomical considerations.

The formation of adventitious structures generally decreased with needle age and distance from the needle base. A comparative anatomical study on needle explants of different developmental stages was therefore carried out. The layer or layers in which cell divisions were initiated and which later gave rise to adventitious structures was also investigated.

Microscopic comparison of the three developmental stages of needle explants studied (I, 3-5 mm; II, 10 mm; and III, 15 mm long needles, Fig. 4) showed marked anatomical differences among them, especially concerning the early differentiation of the abscission zone. On needles of stage I the abscission zone was weakly-developed, comprising a band of about 2-3 prominently



Fig. 4. Needle explants of *Picea abies* at the three different developmental stages used in the experiments.

nucleated, still dividing cells. In the 10 mm long needle (stage II) the cells of the future abscission zone had elongated considerably and the hyaline cells had commenced their differentiation. The organogenic response of needles of stages I and II was obtained proximal as well as distal to the abscission zone. Although the layers of the abscission zone had not yet reached maturity in the needles of stage III, the different cells composing it were now clearly distinguishable: hyaline cells, tooth cells and sclerenchyma. In this case the organogenic response was limited to the proximal tissue, that is, that of the peg-like cushion.

When needles of stage II were transferred to a bud-induction medium, divisions were initiated distal to the abscission zone (on the needle itself) in both epidermal and hypodermal layers, and when advancing higher up on the needle, often restricted to the epider-

mis only. The cells of the hypodermis had presumably differentiated basipetally into fibres and lost their capability of undergoing cell division. Mitotic activity was also observed in subsidiary cells of the stomatal complexes.

When carefully removed, the explant consisted of the needle itself plus the peg-like cushion of axillary meristematic tissue proximal to the abscission zone. Bud primordia were frequently induced very early in this region, the initial divisions being initiated in the cells of the epidermis as well as in subepidermal layers.

III Morphogenesis in dormant embryonic shoots of *Picea abies*: Influence of the crown and cold treatment.

The crown is described as a layer of four to seven cells of homogeneously thickened collenchyma at the base of the dormant embryonic shoot of *Picea abies*. The presence of the crown was found to stimulate the growth of the shoot significantly.

Embryonic shoots retaining the crown and given a 3-h-pulse-induction treatment with 125 μ M BA responded better than those lacking it. This was valid both for the number of explants producing adventitious buds as well as the number of shoots produced per explant. In one of a number of clones tested all the explants plus crown produced adventitious shoots, whereas among

explants excluding it only 31% responded. Corresponding numbers for adventitious shoots produced per explant were 10 for plus-crown explants compared with 3 for minus-crown explants.

The development of buds also occurred earlier in explants plus crown, sometimes nearly twice as early as explants minus crown.

Exposure to cold treatment always decreased the ability to induce adventitious buds as well as the subsequent development of these buds. On the other hand, the shoots showed increasing growth in length after cold treatment. This could perhaps be the result of an altered level of growth regulators induced by chilling. Also the survival of the embryonic shoots was negatively effected by cold treatment, a phenomenon that was partly counteracted by BA treatment but not influenced by either the absence or presence of the crown.

A number of anatomical changes were identified in the cultured explants. In the centre of the originally homogeneous collenchymatous crown, a band of sclerenchymatous cells, 1-2 cells in width, differentiated after 3-4 weeks in culture and after 6 weeks the whole crown comprised a sclerenchymatous layer continuous with the innermost bud scales.

When embryonic shoots were cultured minus the crown, a tissue identified as a possible wound periderm formed at

the excision surface. This was probably the result of mechanical wounding at the time of excision.

Up to five adventitious buds could be induced from a single needle primordium. Vascular connections were established between the newly-formed shoot and the original explant. The anatomy of these connections is very complex, especially where the needle vascular tissue is conjoint with that of the stem tissues of the shoot.

However, marked differences in response between clones were observed, serving as a reminder of the influence of genetic factors.

To sum up, it is evident that when attempting adventitious shoot regeneration in vitro, the retention of the crown on the embryonic shoot leads to a higher yield.

IV Effect of the crown on uptake and transport in embryonic shoots of *Picea abies*.

The possible role of the crown as a barrier to or filter against transport of some inorganic ions $K^+(^{86}Rb)$, $[^{35}S]$ -sulphate, $[^{32}P]$ -phosphate, and the plant growth regulator IAA supplied as $[^{14}C]$ -IAA was investigated.

When $[^{14}C]$ -IAA was supplied at the base of the embryonic shoot, that is for acropetal transport, the main fraction of ^{14}C was bound in the crown region and/or in

the tissues below it. The crown also effectively prevented ^{14}C -transport basipetally. In experiments with embryonic shoots minus crown the receiver contained 8-10 times more ^{14}C than did the receiver for embryonic shoots plus crown. It should also be pointed out that the content of ^{14}C in the receiver agar blocks was significantly higher after basipetal supply than after acropetal supply of $[^{14}\text{C}]$ -IAA. The transport pattern of ^{32}P was affected in essentially the same way as described above for ^{14}C .

Experiments performed at 5° and 20°C indicated the metabolic dependence of these transport processes. This was true for both ^{14}C and ^{32}P , although the transport of ^{14}C was affected to a greater extent than for ^{32}P .

CONCLUSIONS

The results presented in this dissertation can be summarized as follows:

- I Phyllomorph regeneration of shoots in vitro from *Picea abies* is possible.

Pronounced effects on organogenesis were observed when nanomolar levels of NAA were added in combination with BA to the culture medium. The effect was twofold: pseudobulbils (meristemoids), later differentiating into shoots, were induced acropetally along the needle axis; and shoot

regeneration was stimulated considerably.

- II As the needle differentiates in a basipetal direction, the redifferentiating ability of the cells situated in different positions on the needle explant is modified.

Bud-induction stimuli (BA and NAA), when exogenously applied to a 10-mm-long needle, initiated cell divisions immediately distal to the abscission zone in epi- and hypodermal cells. By contrast, in tissues in a progressively more distal position cell division activity was more and more restricted to the epidermis as differentiation of the hypodermal cells into fibres had already advanced beyond the point of reversion.

The proximal cushion tissue retains its responsiveness, a fact underlining the importance of carefully excising the needle from the shoot when transplanting.

In attempting regeneration of plants from conifer leaves, a critical needle length (age) should not be exceeded.

- III Embryonic shoots can serve as a possible explant source in clonal micropropagation of *Picea abies*, particularly if the explant is excised beneath the crown.

The retention of the crown, comprising collenchyma and of which the function is unclear, clearly stimulated adventitious shoot formation. Both the number of explants responding and the yield of adventitious buds per shoot were positively affected. Cold treatment had no positive effects on induction of adventitious buds. The stimulated shoot growth and needle elongation observed on explants cultured in the presence of the crown, indicates that the crown might act as a barrier to basipetal transport and loss of compounds into the medium.

IV It is evident that the crown at this stage of development prevents transport of substances such as $[^{32}\text{P}]$ -phosphorus and $[^{14}\text{C}]$ -IAA in both acropetal and basipetal directions.

Plant growth-regulating substances produced in the embryonic shoot might be prevented from leaking into the medium, thereby influencing the pattern of growth. This could perhaps help explain why embryonic shoots cultured in the presence of the crown grew better, and why when cytokinin-treated, they yielded more adventitious buds than did explants lacking it.

It was realized (III) that information on transport and content of growth regulators was important for the

understanding of the various responses obtained. Recent high pressure liquid chromatographic analyses (Sandberg et al. 1981, Odén 1982) of 0-9 week cold-treated embryonic shoots indicated that the content of IAA sharply increased after 6 weeks of cold-treatment. The content of ABA on the other hand decreased only slightly (Fig. 5). It is hoped that these findings will be helpful in explaining why the embryonic shoots respond differently after cold-treatment, leading to a decreased ability to form adventitious shoots and an increased growth in length of the embryonic shoot explant.

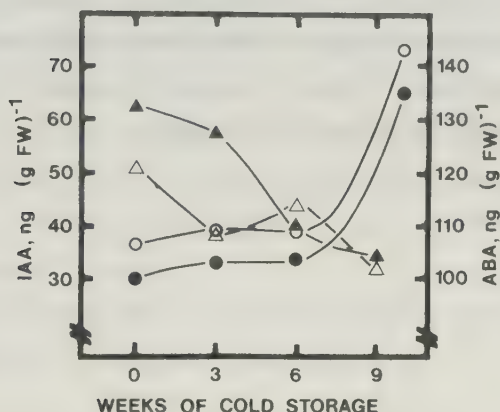


Fig. 5. Content of IAA and ABA in embryonic shoots after various periods of cold-treatment at $4\pm 1^{\circ}\text{C}$.

○, IAA clone A; ●, IAA clone B; △, ABA clone A; ▲, ABA clone B. Each sample consisted of 26-43 embryonic shoots.

A major conclusion can be drawn from the results of the research reported in this dissertation: knowledge of the anatomy and ontogeny of structural changes that occur during development and maturation of the embryonic shoot and needle allows for a more rational selection of appropriate explant material for tissue culture work. Physiological response in terms of adventitious bud induction and subsequent shoot development is more accurately predictable and reproducible.

With a better understanding of the factors which control response, especially in the embryonic shoot, these investigations could conceivably form a basis for future work on rejuvenation of superior Norway spruce trees. The most immediate future problem awaiting solution is that of whole-plant regeneration of the buds induced adventitiously on the embryonic shoot.

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